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**Biological-based Assay for the Screening of
Neutralizing Antibodies to Toxins
(Final Report)**

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FINAL REPORT FOR PROJECT ENTITLED:

Biological-based Assay for the Screening of Neutralizing Antibodies to Toxins.

**Contract No. W7702-5-R543/01=XSG
SSC File No. XSG95-00136-(607)**

(Period from August 16, 1996 to July 31, 1998)

**Submitted to: Science and Professional Services
Western Region, Public Works and
Government Services Canada**

and

**Dr. John Chewonogrodsky
Medical Countermeasures Section,
Defence Research Establishment Suffield**

**Submitted by: T.L. Furukawa-Stoffer, Researcher,
R. J. Weselake, Professor,
University of Lethbridge**

**M. Mah, QAO
D. Mah, Projects Director
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Executive Summary

Three mouse monoclonal hybridoma clones [designated HRF4, HHRD7 and HHRD9], were identified by their resistance to the cytotoxic effects of ricin when cultured in the presence of ricin. All were found to produce neutralizing antibody to ricin toxin. These hybridoma clones were selected and identified from monoclonal hybridomas obtained by cell fusion of myeloma cells and splenomic lymphocytes from ricin-hyperimmunized mice. To varying degrees, these monoclonal antibodies were able to protect myeloma cells challenged with ricin in tissue culture as well as mice challenged with co-injections of ricin.

Results from this work suggests that in the preparation of neutralizing monoclonal antibodies to a cytotoxic antigen, selection using the toxin itself is an effective technique for obtaining toxin-neutralizing monoclonal antibodies. In cases where the cytotoxins are not effective against the monoclonal hybridoma cells themselves, a susceptible "indicator" cell line may be co-cultured with the monoclonal hybridomas during the selection process.

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Figure 2. Determination of antibody titre of hybridoma supernatants producing antibodies directed against ricin by an ELISA procedure.

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Table 1. Determination of ricin toxicity in mice.

Table 2. Number of mice surviving following treatment with 0.5 ug of ricin.

Abbreviations:

BSA	Bovine serum albumin
CDR	complementarity determining region rf. antibody peptide domains involved in antigen-binding
CPE	cytopathic effect
DMEM	Dulbecco's Modified Eagle Medium
ELISA	enzyme-linked immunosorbant assay
FBS	Fetal bovine serum
HAMA	human anti-mouse antibody response
HAT	hypoxanthine, aminopterin, thymidine -supplement
HHRD7	clone designation-hybridoma HAT+ricin-selected well D7
HHRD9	clone designation-hybridoma HAT+ricin-selected well D9
HRF4	clone designation-hybridoma ricin-selected well F4
HRP	horse-radish peroxidase.
LD50	Lethal Dosage 50% survival
μL	microliter
PBS	Phosphate buffered saline
RTA	A-chain of ricin
RTB	B-chain of ricin

1. Introduction

The seeds of the castor bean plant (*Ricinus communis*) contain an extremely toxic protein known as ricin. This is one of the most potent cytotoxins known to man. Ricin is composed of two subunits, commonly referred to as the A-chain (RTA) and B-chain (RTB), which are linked by a disulfide bond (1). The B-chain contains recognition sites for galactose residues and is responsible for binding to galactosyl-containing receptors on cellular membranes (1-3). The B-chain facilitates the entry of the A-chain into cells, whereas the A-chain enzymatically inactivates the 60S subunit of eukaryotic ribosomes (4). The modified ribosomes result in an irreversible inhibition of protein synthesis which subsequently leads to cell death.

There has been extensive research in the use of ricin for the development of immunotoxins to be used for immunotherapeutic applications (5). This involves administering a conjugate of RTA and a monoclonal antibody, which recognizes an antigen from cancer cells. The monoclonal antibody in turn delivers the toxin to the cancer cell. Recent applications of this biotechnology have involved the use of conjugates directed against antigen from Hodgkin tumors (6-7) and breast-cancer cell lines (8).

Medical research involving ricin requires the routine handling of large quantities of the toxin. Ricin may also represent a potential biological-warfare agent or terrorist weapon (Cherwonogrodzky, personal communication). Thus, the potential for exposure of humans to ricin has increased. Due to ricin's extreme toxicity and the lack of any effective treatment for its toxic effects, the development of monoclonal antibodies directed against ricin has become investigated intensively (9-13). Immunizations have been conducted with modified forms of ricin, RTA and RTB.

In developing a therapeutic treatment for ricin intoxication using antibodies, it is imperative that these antibodies are capable of neutralizing the effects of a toxic dose of ricin. Recently, a number of studies have demonstrated that animals can be protected by passive immunization (11) and the deleterious effects of ricin can be prevented if antibodies are given following the injection of lethal doses of ricin (14-15). The high *in vivo* toxicity of ricin precludes its use as an immunogen for the elicitation of protective immunity (16). Protection against the toxic effects of ricin, however, has been achieved through the use of anti-ricin antibodies and anti-idotypes. Chanh *et al.* (17) have developed an anti-ricin monoclonal antibody (termed BG11-G2) which

recognized a conformational epitope of native ricin. This monoclonal antibody was used to passively protect naive mice against ricin intoxication. More recently, anti-idiotypes, which mimic ricin, have been generated by immunizing rabbits with monoclonal anti-BG11-G2 antibodies (16). Mice which received injections of the anti-idiotypic developed resistance to ricin suggesting an anti-idiotypic-induced priming of an enhanced protective immune response to ricin.

A disadvantage to the extensive production of anti-ricin antibodies, is the large numbers of hybridomas which must be screened in order to select a hybridoma clone that specifically produces antibodies to ricin. Various techniques have been used to select hybridomas which produce monoclonal antibodies that block the effect of ricin. One approach to selection has involved assessing the impairment of protein synthesis in hybridomas challenged with various concentrations of ricin (13, 18). Wright (9) has screened hybridomas for neutralizing monoclonal antibodies to ricin by challenging normal diploid human embryonic lung fibroblasts which were osmotically injected with anti-ricin monoclonal antibodies. Anti-ricin monoclonal antibodies provided a 140-fold protection against the effects of ricin. Derbyshire and Wawrzynczak (19) screened monoclonal antibodies for their ability to mediate the toxic effects of RTA against human small cell lung cancer using an indirect assay of immunotoxin cytotoxicity. The development of time-efficient and cost-effective screening procedures for the selection of hybridomas would be of great benefit when monoclonal antibodies are required. In the current study, we have developed a screening procedure whereby hybridomas producing neutralizing antibodies to ricin survive in the presence of ricin. These anti-ricin antibodies were also shown to neutralize the toxic effects of lethal doses of ricin when injected with ricin into mice. As well, the antibodies enhanced the viability of Sp2/1IL6 myeloma cells grown in the presence of ricin.

2. Materials and Methods

Chemicals

Dimethylsulfoxide, polyethylene glycol, and ricin (RCA60) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), L-glutamine, hypoxanthine, aminopterin, and thymidine supplement (HAT), minimum essential medium (MEM) sodium pyruvate, and penicillin-streptomycin were purchased from GIBCO-BRL, Canadian Life Technologies (Burlington, ON, Canada). Horseradish peroxidase substrate kit was purchased from Bio-Rad Laboratories (Mississauga, ON, Canada).

Myeloma Cells

Myeloma Sp2/1IL6 cells were obtained from the Southern Alberta Cancer Research Centre Hybridoma Facility (SACRC Hybridoma Facility) and were maintained in 80 cm² tissue culture flasks (Gibco-BRL, Canadian Life Technologies, Burlington, ON, Canada) in DMEM supplemented with 15% (v/v) heat-inactivated FBS, 1.0 mM MEM sodium pyruvate, 2.0 mM L-glutamine and 1% (v/v) penicillin-streptomycin. Cells were cultured in a humidified atmosphere of 5% (v/v) CO₂ at 37°C and aseptically divided into fresh DMEM-FBS media every 4 days.

Animals

Balb/C mice (male, 42-48 days old) were obtained from Charles River Canada (Montreal, QC, Canada) and were housed in cages and fed and watered *ad libitum*.

Determination of ricin toxicity in mice

Groups of 3 mice received intraperitoneal injections of 100 µL phosphate-buffered saline (PBS) containing 0, 0.02, 0.04, 0.1, 0.2, 0.3, 0.4, 0.5, 2.0 or 10 µg ricin toxin. The mice were monitored for 4 days following injection.

Immunizations

Immunizations were performed using intraperitoneal injections. Each mouse received a sub-lethal dosage of 0.1 µg ricin in PBS. The mice received a total of 5 injections. Mice were bled from the tail vein to acquire antisera for determination of antibody titre. Mice subjected to a

spleenectomy for monoclonal antibody production were given a booster injection of 0.1 µg ricin intravenously through the tail vein, 3 days prior to surgery.

Cytotoxicity assay

DMEM-FBS media supplemented with 500 ng ricin/mL was prepared and a series of dilutions in a final volume of 125 µL were made in 11 wells of a 96-well microtitre plate (Gibco-BRL, Canadian Life Technologies, Burlington, ON, Canada). Concentrations ranged from 0.49 ng ricin/mL to 500 ng ricin/mL. DMEM-FBS media containing no ricin was used as a control. The myeloma cells were diluted to 5×10^5 cells/mL in 12 mL DMEM-FBS media and 200 µL was aliquoted into 48 wells. In duplicate, 50 µL of each concentration of ricin was added to the myeloma cells (24 wells). Final concentrations of ricin ranged from 0 to 100 ng/mL. The remaining 24 wells were control wells. The cells were cultured for 2 days and the surviving cells were visualized with an Olympus Tokyo inverted microscope (Olympus Optical Co., Ltd., Tokyo, Japan) at 10X magnification. The cytopathic effect (CPE) was determined by comparing the number of surviving cells to cells cultured in the absence of ricin.

Hybridoma production and screening for hybridomas producing neutralizing antibodies

The spleens from two mice which received 5 injections and a booster injection of 0.1 µg ricin were removed and the spleen cells were fused to Sp2/1IL6 myeloma cells according to Harlow and Lane (20). The fused cells were plated onto 96-well microtitre plates in DMEM-FBS media supplemented with HAT, HAT + 0.4 ng ricin/mL, or 0.4 ng ricin/mL. Hybridoma growth was monitored for 7 - 10 days. Supernatants from hybridomas which survived ricin exposure were tested using an enzyme-linked immunoassay (ELISA) procedure for antibody titre. Hybridoma cell lines producing anti-ricin antibodies were transferred to 6-well tissue culture plates containing DMEM-FBS media and cultured as described for the myeloma cells. Cell lines were cultured for 7 days then transferred to 25 cm² tissue culture flasks.

ELISA assay

A 96-well plate (Nunc Immunosorp, Gibco-BRL, Canadian Life Technologies, Burlington, ON, Canada) was used. Wells received 200 µL carbonate buffer, pH 9.5, containing

0.25 µg ricin overnight at 4°C. The wells were washed 5 X with 200 µL PBS buffer containing 0.1% (w/v) bovine serum albumin (BSA) and 0.05% (w/v) Tween 20 and were blocked with 200 µL PBS buffer containing 5% (w/v) skim milk powder, 0.1% (w/v) BSA and 0.05% (w/v) Tween 20 for 1 hr at 37°C. The wells were washed 5 X with 200 µL washing buffer and were incubated with 100 µL hybridoma supernatants for 1 hr at 37°C. The wells were washed 5 X with 200 µL washing buffer. Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG was added and the wells were incubated for 1 hr at 37°C. The wells were washed 5 X with 200 µL washing buffer and were developed using the ABTS (2,2'-azino-di-3-ethyl-benzthiazoline-6-sulfonic acid) HRP substrate for 10 min at 25°C. The optical density of the substrate was read at 415 nm in a Titertek Multiskan ELISA reader (Titertek Instruments Inc., Huntsville, AL, USA).

Propagation of myeloma cells in the presence of ricin and anti-ricin antibodies

Hybridoma supernatants HRF4 (previously shown to produce antibodies which tested strongly positive for ricin in ELISA assays), HHRD7 (previously shown to produce antibodies which tested moderately positive for ricin in ELISA assays), and HHRD9 (previously shown to produce antibodies which tested slightly positive for ricin in ELISA assays) were serially diluted with DMEM-FBS media in a final volume of 125 µL in 11 wells of a 96-well microtitre plate. Concentrations ranged from 2-fold to 1024-fold diluted supernatant. DMEM-FBS media containing no hybridoma supernatant was used as a control. The myeloma cells were diluted to 5×10^5 cells/mL in 25 mL DMEM-FBS media and 200 µL portions were dispensed into 84 wells. In duplicate, 25 µL of DMEM-FBS media supplemented with 4 ng ricin/mL were combined with 25 µL of each dilution of hybridoma supernatant and added to the myeloma cells (72 wells). Final concentrations of hybridoma supernatant ranged from 0- to a 10 240-fold dilution. The remaining 12 wells were control wells. The cells were cultured for 2 days and monitored for a CPE.

Neutralization of ricin by anti-ricin antibodies in mice

Two mice received an intraperitoneal injection of 200 µL containing 100 µL anti-ricin serum (obtained from DRES) combined with 100 µL PBS buffer containing 0.5 µg ricin. Two additional mice received 200 µL which contained 100 µL hybridoma supernatant HRF4 (previously shown to produce antibodies which tested strongly positive for ricin in ELISA assays)

combined with 100 μ L PBS buffer supplemented with 0.5 μ g ricin. Two mice were administered 200 μ L which contained 100 μ L hybridoma supernatant HHRD9 (previously shown to produce antibodies which tested slightly positive for ricin in ELISA assays) combined with 0.5 μ g ricin in 100 μ L PBS buffer. One mouse received 100 μ L PBS buffer containing 0.5 μ g ricin. The mice were monitored for 4 days following injections.

In a second neutralization assay, six groups of three mice were given an intraperitoneal injection of 200 μ L containing 100 μ L 0.5 μ g (2X LD₅₀) ricin prepared in PBS buffer and 100 μ L of either DMEM-FBS media, 20-fold or 100-fold diluted pre-immune serum, 20-fold or 100-fold diluted antiserum, HRF4 hybridoma supernatant, HHRD7 hybridoma supernatant or HHRD9 hybridoma supernatant. As a control, one group of five mice received a 200 μ L injection containing DMEM-FBS media and PBS buffer. The mice were monitored for seven days following injections.

Table 1. Determination of ricin toxicity in mice.

<u>Dosage of Ricin (μg)</u>	<u>Number of surviving mice</u>	<u>Number of dead mice</u>
0	6 ^a	0
0.02	3	0
0.04	3	0
0.10	6	0
0.20	6	0
0.30	0	6
0.40	0	6
0.50	0	6
2.0	0	3
10.0	0	3

^aGroups of 3 or 6 Balb/C mice received intraperitoneal injections of 100 μ L phosphate-buffered saline containing various quantities of ricin. Mice were monitored for 4 days following injections.

3. Results

Toxicity of ricin in mice

Mice were injected with varying concentrations of ricin in order to estimate a dosage of toxin at which 50% of the mice would die (Table 1). Mice which received 0.2 µg ricin or less per mouse survived. Their fur was rough, and their level of activity and food consumption substantially decreased, but 10 days following injections, the mice appeared "healthy" again. Mice which received 2.0 and 10.0 µg ricin died within 2 days following administration of ricin toxin. The mice appeared lethargic and in respiratory distress in the days preceding their death. Mice which received 0.3, 0.4, and 0.5 µg ricin appeared "sickly" following injections and all 3 mice in each group died within 4 days. The control mice (0 µg ricin) did not appear to suffer any effects following injections. The dosage of ricin resulting in death of 50% of the mice was determined to be between 0.2 and 0.3 µg ricin per mouse. Subsequently, mice which received doses less than 0.2 µg ricin were further immunized for antibody production.

Cytotoxicity assay

Myeloma cells cultured for 2 days in DMEM-FBS media without ricin displayed well established cell growth in each well. Cells grown in the presence of ricin displayed decreased viability as ricin concentration in the surrounding medium increased (Figure 1). CPEs of about 90% and 70% occurred with ricin concentrations of 0.2 and 0.1 ng/mL, respectively.

Hybridoma production and screening of hybridomas producing anti-ricin antibodies

Hybridomas are generally cultured in media supplemented with HAT and as a result, only hybrid cells will remain viable in culture. Myeloma cells cultured in the presence of DMEM-FBS media supplemented with HAT did not survive following 2 days of growth in HAT (data not shown). Screening of large numbers of hybridomas was facilitated by the use of DMEM-FBS media supplemented with 0.4 ng ricin/mL. Hybridomas were cultured in the presence of media supplemented with HAT, HAT + 0.4 ng ricin/mL or 0.4 ng ricin/mL. The development of hybridoma colonies was monitored in 144 wells of two cell culture plates, for each supplemented medium. A large number of hybridomas (approximately 45) were produced when cultured in the

presence of media supplemented with HAT. Only 2 hybridomas were produced when cultured in the presence of media supplemented with HAT + 0.4 ng ricin/mL and 1 hybridoma colony was produced when cultured in the presence of media supplemented with 0.4 ng ricin/mL. Selection of the hybridoma lines producing antibodies against ricin was made possible by monitoring the propagation of clones in the presence of ricin. In order to confirm that these hybridomas were producing anti-ricin antibodies, a number of hybridomas from media supplemented with HAT and the three hybridomas from media supplemented with HAT + ricin or ricin were tested for ricin antibodies using an ELISA procedure. This screening procedure resulted in the selection of the 3 hybridoma lines that were cultured in the presence of ricin.

The titres of the antibodies determined by ELISA are depicted in Figure 2. A hybridoma from media supplemented with HAT + ricin (HHRD7) and one hybridoma from media supplemented with ricin (HRF4) tested positive for producing antibodies directed towards ricin. The HRF4 supernatant resulted in a strong response to ricin when compared to control media, indicating the presence of anti-ricin antibodies. Antibody binding, as indicated by increased absorbance at 415 nm, was observed using a 800-fold dilution of hybridoma supernatant. Hybridoma, HHRD7 resulted in a weaker response when compared to control media. In this case, antibody binding was observed at a 200-fold dilution of hybridoma supernatant. Supernatant from hybridoma HHRD9, cultured in media supplemented with HAT + ricin, showed no increase in absorbance values above that of the control.

The screening of hybridomas in the presence of ricin facilitated the identification of ricin-resistant clones which were capable of producing neutralizing antibodies to the toxin. Hybridoma line HRF4, was further cultured in DMEM-FBS media without ricin. The hybridoma line was effectively propagated when a small portion of the cells were reintroduced into DMEM-FBS media containing 0.4 ng ricin/mL. After 4 days, the cells which were previously selected in the presence of ricin, and had subsequently been maintained in the absence of ricin, were able to grow efficiently when placed back into media supplemented with ricin.

Protection of myeloma cells using anti-ricin antibodies

Hybridoma supernatants containing antibodies directed against ricin protected myeloma cells against the cytotoxic effects of ricin intoxication (Figure 3). The ricin concentration was 0.4

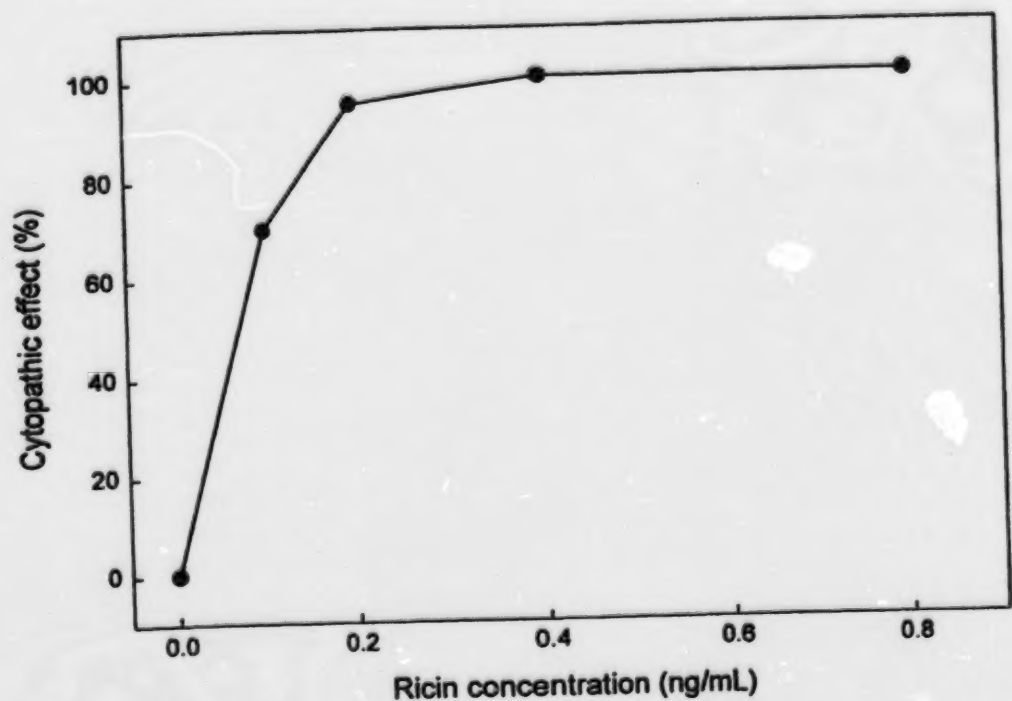


Figure 1. Cytopathic effect (CPE) of SP2/1IL6 myeloma cells cultured in the presence of ricin. Myeloma cells diluted to 5×10^5 cells/mL in 12 mL DMEM-FBS media were added to the wells of a 96-well plate containing varying concentrations of ricin in DMEM-FBS media. Cells were cultured for 2 days and monitored for CPE.

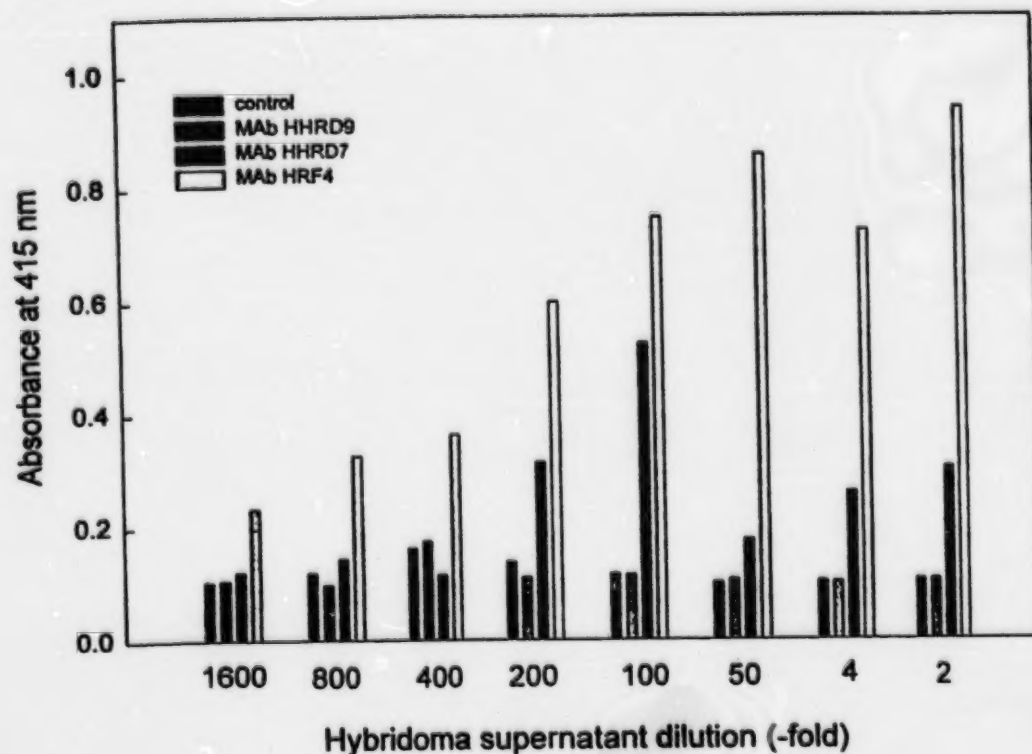


Figure 2. Determination of antibody titre of hybridoma supernatants antibodies directed against ricin by an ELISA procedure. Micro-titre plate wells were coated with 0.25 μ g ricin and 100 μ L of hybridoma supernatant diluted with DMEM-FBS media was added to the wells. The ELISA plate was developed using horseradish peroxidase-conjugated rabbit anti-mouse IgG/ABTS substrate and measured at 415 nm. DMEM-FBS media containing no hybridoma supernatant was used as the negative control. Data represents the means of duplicate assays.

ng/mL. A 95% CPE was demonstrated in the case where myeloma cells were incubated with ricin without antibody. A CPE of 40% was demonstrated using a 10-fold dilution of HRF4 supernatant. In contrast, a CPE of 80% was demonstrated using a 10-fold dilution of hybridoma supernatant HHRD9. Hybridoma HRF4 was previously shown to produce higher titre antibodies than HHRD9 (Figure 2).

Neutralization of toxic effects of ricin by anti-ricin antibodies in mice

Passive immunization with antibodies directed against ricin protected mice against the deleterious effects of ricin intoxication. In an initial neutralization experiment, mice which received an intraperitoneal injection of anti-ricin serum (obtained from DRES) combined with a lethal concentration (0.5 µg) of ricin, survived exposure to ricin. The fur of the mice appeared rough for 3 days following injections, but returned to normal shortly thereafter. A similar response was observed in mice which received an injection of hybridoma supernatant (HRF4) containing anti-ricin antibodies combined with a highly lethal dose of ricin. The hybridoma supernatant from line HHRD9, containing a low titre anti-ricin antibodies, also protected mice from the toxic effects of ricin. Only the control mouse, which received an injection of 0.5 µg ricin without the anti-ricin antibodies, died from ricin intoxication. All mice which received antibodies and a lethal dose of ricin survived for 5 months after which they were euthanized.

In a second neutralization experiment, mice which received pre-immune serum combined with 0.5 µg ricin, or DMEM-FBS media combined with 0.5 µg ricin, died within 48 hours following injections (Table 2). Two out of three mice which received an injection of anti-ricin serum combined with 0.5 µg ricin, died after 72 hours following injections. One mouse which received HHRD9 hybridoma supernatant combined with 0.5 µg ricin died after 120 hours following injection. This particular mouse may have died as a result of fighting between the other mice in its cage as its head was missing. This may have been more of a sign of aggression than as a result of ricin intoxication. All the remaining mice survived the toxic affects of ricin with protection from antibodies directed against this toxin.

Table 2. Number of mice surviving following treatment with 0.5 μ g ricin.

Experimental Group	No. Mice	1 Day	2 Day	3 Day	4 Day	5 Day
PBS control	5 ^a	5	5	5	5	5
Ricin + Pre-sera (1/20)	3	3	3	1	0	
Ricin + Pre-sera (1/100)	3	3	1	0		
Ricin + 10% DMEM	3	3	0			
Ricin + Antisera (1/20)	3	3	3	3	3	3
Ricin + Antisera (1/100)	3	3	3	1	1	1
Ricin + HRF4	3	3	3	3	3	3
Ricin + HHRD7	3	3	3	3	3	3
Ricin + HHRD9	3	3	3	3	3	2

^aGroups of three or five mice were given an intraperitoneal injection of 200 μ L containing 100 μ L 0.5 μ g ricin prepared in PBS buffer and 100 μ L of either DMEM-FBS media, pre-immune serum, antiserum, HRF4 hybridoma supernatant, HHRD7 hybridoma supernatant or HHRD9 hybridoma supernatant. As a control, a group of five mice received a 200 μ L injection of DMEM-FBS media combined with PBS buffer.

4. Discussion

Screening for neutralizing antibodies to toxins is generally a time consuming and labor intensive process. The development of a simple, biological-based procedure for the selection of hybridomas producing neutralizing antibodies to cellular toxins would significantly reduce the amount of time required to screen large numbers of hybridomas. We utilized a procedure that involved culturing hybridomas in the presence of ricin in order to select for hybridomas which produced antibodies directed against ricin. Two hybridomas, HRF4 and HHRD7, which were effectively cultured in the presence of ricin produced high and moderately high titre antibodies against ricin, respectively, based on ELISA assays. Thus, the hybridoma cells were able to survive in the media supplemented with ricin as a result of the production of antibodies which were directed against ricin. These hybridomas were subsequently maintained in the absence of ricin and were able to grow efficiently when placed back into ricin. This indicated that the hybridoma cell lines developed were still capable of producing neutralizing antibodies to ricin after extensive sub-culturing.

In order to obtain a maximum immunological response, it was important to conduct immunizations of mice with quantities of ricin as close as possible to the lethal dose of the toxin without killing the mice. In our determination of the toxicity of the ricin used in this study, it was observed that all the mice which had received 0.1 μg ricin survived and those which were injected with 0.5 μg ricin died. Hybridomas with high titre antibodies to ricin, however, were produced as a result of these injections.

In the literature, there appears to be no consensus on the toxicity of ricin in mice. For example, Houston (14) reported an LD50 of 0.4 $\mu\text{g}/\text{mouse}$ whereas Lemley and Wright (21) reported an LD50 of 0.08 $\mu\text{g}/\text{mouse}$. The LD50 for ricin in mice varies between preparations of ricin and different strains of the mice show different sensitivities to ricin (14). In our passive immunization experiments with mice, we used 0.5 μg ricin/mouse because this was the concentration of ricin which was lethal for all the mice. Monoclonal antibodies, HRF4, HHRD7 and HHRD9, were able to protect mice from the deleterious effects of ricin intoxication when the antibodies and toxin were injected simultaneously into mice. The antibodies also enhanced the viability of myeloma cells grown in the presence of ricin. The neutralization effect provided by these antibodies suggested that the antibodies may be useful in the further development of an

immunotherapeutic treatment for ricin intoxication in humans.

A number of researchers have shown that rabbit or mouse anti-ricin antibodies prolonged the survival of mice given intraperitoneal injections of lethal doses of ricin (14, 16-17, 22). As well, Hewetson *et al.* (23) demonstrated that an aerosol challenge of ricin could be neutralized if mouse anti-ricin antibodies were administered intravenously to mice prior to or following ricin administration. Rabbit anti-ricin antibodies were also shown to be effective at rescuing mice from ricin intoxication when lethal doses of ricin had been injected subcutaneously (15). Anti-ricin antibodies were equally as effective in prolonging the survival of mice when ricin was administered by either intracerebral injections or intravenous injections (15). Mice passively immunized by a protective anti-ricin monoclonal antibody, then challenged with ricin, were shown to be protected from the effects of lethal doses of ricin (21, 24).

In conclusion, a biological-based screening technique was developed for identifying hybridomas which produced anti-ricin antibodies. This approach may prove to be useful in screening for monoclonal antibodies directed against other cellular toxins. The neutralizing antibodies developed in this study may lead to the identification of anti-ricin monoclonal antibodies with novel paratopes that could prove valuable in epitope-mapping of ricin. The antibodies could also prove useful in physiological studies of ricin action and in monitoring exposure to ricin. Humanized anti-ricin monoclonal antibodies would be of potential therapeutic use in providing immunity to the toxin in a warfare or terrorist situation. Humanized anti-ricin monoclonal antibodies may also be useful as antidote for children who accidentally ingest castor beans and for researchers and industrialists who may be exposed to relatively large quantities of ricin in the form of an aerosol.

5. Summary and Recommendations

Active immunization of using ricin vaccines would play an important prophylactic role in protecting individuals from hazardous levels of environmental ricin. In situations where individuals are non-immunized or immunized individuals are exposed to overwhelming levels of ricin toxin, passive immunization with neutralizing antibody may be a useful medical intervention. Valuable ricin-neutralizing mouse monoclonal antibodies have been obtained in this work which can be used to both further development of (1) effective vaccines or (2) humanized IgG antibody fragments for medical therapy.

Design of a recombinant vaccines against multiple toxins or pathogens.

Ricin-neutralizing antibodies could be used to determine the neutralization epitopes on ricin toxin by various epitope-mapping techniques. It would be important to determine if all three neutralizing monoclonal antibodies are directed against a single ricin neutralization epitope or whether there is more than one ricin neutralization epitope. Identification of ricin-neutralization epitopes would allow these ricin epitope domains to be genetically cloned as a safe recombinant vaccine. If similar work is successful in identifying the neutralization epitopes of other toxins, viruses or pathogen; it may be possible to eventually design multi-purpose recombinant vaccines against various BW agents.

Humanization of neutralizing anti-ricin monoclonal antibody for passive immunization..

To reduce the HAMA response (human anti-mouse antibody response) valuable therapeutic mouse monoclonal antibodies can be humanized. This can be achieved to varying degrees by CDR-grafting (i.e. transfer of antibody peptide segments [CDR regions] involved in antigen-binding from mouse mAb to a human IgG framework) or generation of chimeric human/mouse monoclonal antibodies (i.e. fusion of the mouse VH/VL combining site to human IgG constant regions) (25, 26, 27, 28). Work to humanize the anti-ricin neutralizing monoclonal antibodies would first involve the genetic cloning of the IgG Lc and Hc from each of the anti-ricin monoclonal hybridoma clones. The antibody DNA genes would be sequenced and the amino acid sequence of the antibody would be determined [in particular the CDR- regions]. Synthetically generated DNA gene sequences encoding the antibody CDR regions could be then cloned into corresponding regions in

a cloned human antibody gene. Because of economic cost and technical complexities, humanization should be performed only on extremely useful mouse mAb. Often this means that clinical evaluations must be based upon the use of the mouse monoclonal antibody which does not necessarily reflect the performance of an equivalent "humanized" antibody.

Selection of neutralizing antibodies to other cytotoxic antigens.

Selection of neutralizing monoclonal antibodies to a cytotoxic antigen using the toxin itself has been demonstrated to be an effective technique in isolating monoclonal hybridoma clones which produce neutralizing antibody. This technology may also be readily adapted to obtain neutralizing monoclonal antibodies against other toxins (eg. botulinum toxin, anthrax) or pathogenic viruses (eg. WEEV, EEEV). In cases where the cytotoxins are not effective against the monoclonal hybridoma cells or cells are not permissive to viral infection themselves, a susceptible "indicator" cell line may be co-cultured with the monoclonal hybridomas during the selection process.

6. Summary of Deliverables

The following items have been submitted as deliverables to the Government of Canada.

- 1) Final report in seven hard copies. Sections are formatted to facilitate submission for publication in the journal "Hybridoma".
- 2) Final report and publication pre-print -soft copy on 3.5" computer disk (dos format).
- 3) Hard copy (3) publication preprint for journal "Hybridoma"
- 4) Overhead viewgraphs of final report/publication figures and tables.
- 5) Monoclonal hybridoma cell freezer stocks have been submitted as a deliverable to the Government of Canada (see Appendix A) and have been put into storage at DRES facilities.

7. References

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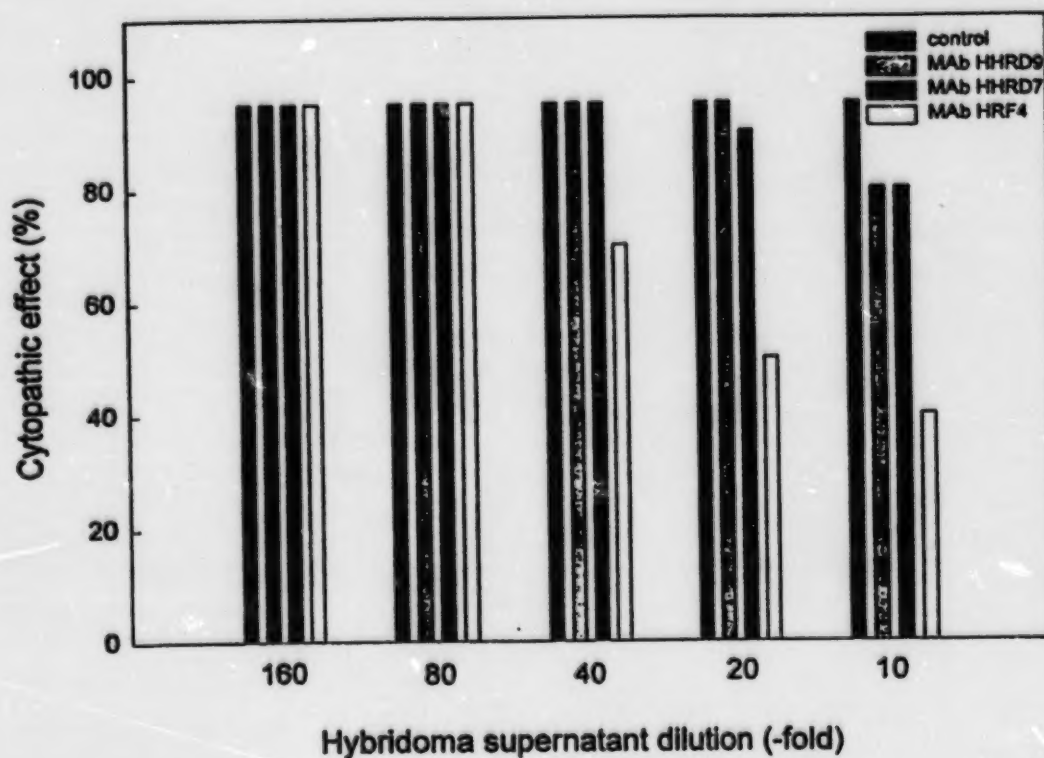


Figure 3. Neutralization of the toxic effects of ricin by anti-ricin antibodies on myeloma cells. Microtitre plate wells containing approximately 1×10^5 SP2/1IL6 myeloma cells received varying dilutions of hybridoma supernatant combined with 0.4 ng/mL ricin. Cells were cultured for 2 days and monitored for ricin-induced cytopathic effects.

Inventory of Deliverables

Scientific Authority: Dr. J. Cherwonogrodsky, Defence Scientist
Contract: W7702-5-R543/01-XSG
Contract Title: BIOLOGICAL-BASED ASSAY FROM THE SCREENING OF
NEUTRALIZING ANTIBODIES TO TOXINS
Location: Liquid Nitrogen Freezer Facility, Tower G, Box G8
Items: Myeloma strains.
Mouse monoclonal hybridomas producing antibodies to ricin.
Date of Delivery: July 22, 1997

<u>Sample</u>	<u>Date(s) Frozen</u>	<u>Quantity*</u>	<u>Description</u>
Sp2IL6	17/6/97	4	Myeloma cell line producing IL6 constitutively. For increased efficiency hybridoma cell fusions.
HRF4	12/6/97	8	Primary hybridoma clone selected with ricin only.
HRF4b	12/6/97	10	Secondary subclone of HRF4 hybridoma clone selected with ricin only.
HHRD7	26/6/97	3	Primary hybridoma clone selected with HAT and ricin.
HHRD9	26/6/97	3	Primary hybridoma clone selected with HAT and ricin.

* Quantity indicates number of freezer vial.

Sample Array:

Since the positions in the box are not numbered a general scheme of how samples are put into the storage box as the user remove it directly, is shown below.

Samples are ordered from left to right as shown in list of deliverables above.

